AN IMMUNOLOGIC STUDY OF BACILLUS INFLUENZAE

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This communication deals with a portion of a joint investigation to determine if any evidence of an immunologic nature could be adduced which would point toward an etiologic relationship between Bacillus influenzae of Pfeiffer and epidemic influenza.

The problem was approached from three standpoints; first, the search for agglutinins for B. influenzae in the serum of convalescents from epidemic influenza; second, the search for complement-fixing bodies in the same material; and third, the study of the immunologic relationships of the various strains of B. influenzae obtained from patients with epidemic influenza. Papers dealing with these three phases of the problem have already appeared.[1] It is the purpose now to record the results of a study of the immunologic relationship of strains of B. influenzae obtained from adults during the course of an attack of epidemic influenza.

The importance of this phase of the problem has been repeatedly emphasized by Park and was clearly in mind at the time these studies were instituted. To quote from Park[2]: "With the coming of the 1918 pandemic we knew that if the influenza bacillus was the initiating cause, we must be dealing with a single type of exalted virulence or toxicity or both, and that unless we could recover influenza bacilli from the large majority of early cases and show a definite antibody relationship between these strains, we could not add evidence as to its being the exciting factor." The occurrence of influenza in epidemic form in the early months of 1920 gave an opportunity for work along these lines, and for testing the results of previous investigators in this field.

HISTORICAL

A review of all the bacteriologic work carried out on cases of influenza during the recent pandemic is considered beyond the scope of

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[1] Utheim, Kirsten: Jour. Infect. Dis., 1920, 27, p. 460; Cooke, J. V.: Ibid., 476; Bell, H. H.: Ibid., 464.

[2] Park, W. H., and Williams, A. H.: Amer. Jour. Pub. Health, 1919, 9, p. 45.

this paper. I shall confine myself to a summary of the studies that have been made in connection with the immunologic relationship of strains of influenza bacilli recovered from cases of epidemic influenza.

Gay and Harris [3] noted that a polyvalent serum produced by immunizing with several strains of B. influenzae failed to agglutinate two strains of this organism that had not been used for immunization, and suggested the existence of separate groups of B. influenzae. Huntoon and Hannum [4] and Roos [5] found evidence of immunologic relationship in the strains they studied. Fleming and Clemenger [6] tested 8 strains and found cross agglutination in one instance only. The most extensive researches in this problem have been those carried out by Valentine and Cooper [7] working under Park's direction. In a study of 181 strains from cases of influenza they found identical strains in different patients in 2 instances only and no evidence of immunologic grouping. On the other hand, Small and Dickson,[8] from a study of 10 strains, concluded that they could distinguish 4 groups on the basis of agglutination and absorption tests. Bell,[1] whose contribution forms part of the joint investigation mentioned, concluded from a study of 36 strains that "the influenza bacillus represents a heterogeneous group of organisms as shown by agglutination and absorption tests" and that identical strains do occur. Furthermore, he was unable to differentiate by those methods between organisms isolated from normal throats during an interval of 2 months prior to the recurrent epidemic of 1920, and those isolated from the throats of influenza patients during that epidemic. More recently, Povitzky and Denny [9] found 4 out of 7 strains obtained from cases of influenzal meningitis and isolated years apart which proved to belong to one immunologic group, and from respiratory cases they found as many as 5 strains from different individuals which belonged to the same group, although they were not able to find any other groups consisting of more than 2 members each. Coca and Kelley,[10] in a study of 18 strains, "isolated in different localities and at different times" found "identities in the cultures only when a probability of personal contact existed."

Apparently no one as yet has succeeded in recovering from cases of epidemic influenza a large proportion of strains of influenza bacilli that are identical in their immunologic relationships.

SOURCE OF MATERIAL

This work deals with 12 strains of influenza bacilli obtained from adults with influenza who were patients in the Barnes Hospital on the service of Dr. Dock. They were obtained during the course of the epidemic of influenza which prevailed in St. Louis during January and February, 1920. They were obtained by nasopharyngeal culture, by

[3] Jour. Infect. Dis., 1919, 25, p. 414.
[4] Jour Immunol., 1919, 4, p. 167.
[5] Jour. Immunol., ibid., p. 189.
[6] Lancet, 1919, 2, p. 869.
[7] Jour. Immunol., 1919, 4, p. 359.
[8] Jour. Infect. Dis., 1920, 26, p. 230.
[9] Jour. Immunol., 1921, 6, p. 65.
[10] Ibid., p. 87.

direct culture of washed sputum or by inoculation of sputum into white mice. They were considered to be influenza bacilli if they were small, pleomorphic bacilli, gram-negative, nonmotile, producing pin-point colonies on blood agar and demanding hemoglobin for their growth. They were isolated originally on 5% rabbit-blood agar and were kept growing on "chocolate agar."

TECHNIC

Rabbits were immunized with suspensions of living influenza bacilli in physiologic salt solution, washed from the surface of "chocolate-agar" plates. Intravenous injections were made at intervals of from 4 to 7 days until preliminary agglutination tests showed an agglutinin titer in the rabbit serum of 1:800 or more for the homologous strain. The animals were then bled and the serum stored in the refrigerator without preservative until used. The animals stood immunization fairly well, only one being lost, and it died shortly after the first injection.

Cross agglutination tests were carried out at 56 C. for 18 hours, preliminary experiments by Dr. Bell having shown that agglutination was sharper when carried out in this manner than if carried out at a lower temperature or for a shorter period of time. The highest dilution at which agglutination was just visible with the naked eye was the one recorded.

Considerable difficulty was encountered in getting some of the strains to remain in suspension. This factor of poor emulsibility seemed to be a constant characteristic of these particular strains, appearing almost uniformly, and was only overcome by a brief preliminary centrifugation of the suspension, thus throwing down the larger clumps and leaving a supernatant fluid which represented a thin suspension of the organisms in question. By this technic spontaneous agglutination was avoided with all the strains but one. This particular strain was so persistent in its spontaneous agglutination that it had to be discarded.

Absorption experiments were carried out at 56 C. for 4 hours, followed by 18 hours' exposure in the refrigerator. If, after this degree of exposure, preliminary tests still showed the presence in the serum of agglutinins for the absorbing strain, the procedure was repeated until all of the agglutinins for that strain were absorbed. For these absorption experiments a heavy suspension of the organisms was added to equal parts of whole serum.

RESULTS

Cross agglutination tests were carried out with all 12 strains and their homologous serums in the manner outlined, and the results are

recorded in table 2. In recording the titer the ultimate dilution is stated. A consideration of this table shows that strains 2, 3, 4 and 8 showed cross agglutination in dilutions ranging from 1 : 1200 to 1 : 3200. Strain 8 was less readily agglutinated than the other three strains. With the exception of these strains, no other evidence of cross agglutination in high dilution occurred. The serums of these 4 strains did not always agglutinate other strains to a like extent. For example, serum 2, serum 4, and serum 8 agglutinated strain 5 in a dilution of 1 : 50 whereas serum 3 agglutinated this strain in a dilution of 1 : 100. Nor were these strains (2, 3, 4 and 8) that gave evidence of cross agglutination always agglutinated to the same degree by heterologous serum. For example, serum 1 agglutinated strains 2 and 3 in a dilution of 1 : 50, but did not agglutinate strains 4 or 8. Other examples could be cited.

TABLE 1

DIRECT AGGLUTINATION OF STRAINS OF INFLUENZA BACILLI WITH HOMOLOGOUS IMMUNE SERUMS

Agglutination at 56 C. for 18 hours

Strains	Normal Rabbit Serum	Immune Serums											
		1	2	3	4	5	6	7	8	9	10	11	12
1	0	800	25	50	0	25	0	0	0	0	100	25	25
2	0	50	3,200	3,200	1,600	50	100	100	1,200	100	50	50	25
3	0	50	3,200	3,200	1,600	50	100	100	1,200	100	100	50	25
4	0	0	3,200	3,200	1,600	50	200	200	1,200	0	100	50	50
5	0	0	50	100	50	1,600	200	0	50	0	50	100	100
6	0	50	25	200	50	50	800	400	100	0	50	100	50
7	0	100	25	100	100	50	200	2,400	0	0	100	25	25
8	0	0	1,600	1,200	1,200	50	100	100	1,600	0	50	25	25
9	0	0	50	100	50	50	200	100	50	1,600	0	100	50
10	0	50	100	100	50	50	0	200	0	0	1,200	100	100
11	0	0	25	200	0	100	200	0	100	0	100	1,600	200
12	0	50	50	50	0	25	200	50	0	0	200	200	800

Among the 12 strains there were 5 (2, 3, 5, 11 and 12) whose serums agglutinated all heterologous strains in varying dilutions, but of these 5 there were only 2 (2 and 3) whose serum agglutinated other strains in high dilution, the limit of agglutination of the other four serums for heterologous strains being 1 : 200.

Except for strains 2, 3, 4 and 8, no other strains gave evidence of falling into a single immunologic group, as determined by the phenomenon of agglutination. These 4 strains, however, as they showed cross agglutination in dilutions ranging from 1 : 1200 to 1 : 3200, one would be inclined to regard as closely related if not identical from an immunologic point of view. To determine the correctness of this view resort was had to absorption experiments. For the absorption experiments the 4 strains giving cross agglutination reactions and 3 other

TABLE 2

CROSS AGGLUTINATION EXPERIMENTS AFTER ABSORPTION

Serum for Strain 2			Serum for Strain 3			Serum for Strain 4			Serum for Strain 8		
Absorbed with Strains	Titrated with Strains	Titer after Absorption	Absorbed with Strains	Titrated with Strains	Titer after Absorption	Absorbed with Strains	Titrated with Strains	Titer after Absorption	Absorbed with Strains	Titrated with Strains	Titer after Absorption
2	2	0	2	2	0	2	2	0	2	2	0
2	3	0	2	3	25	2	3	0	2	3	25
2	4	0	2	4	0	2	4	0	2	4	0
2	5	0	2	5	25	2	5	0	2	5	0
2	6	0	2	6	25	2	6	0	2	6	0
2	8	0	2	8	0	2	8	0	2	8	25
2	9	0	2	9	25	2	9	0	2	9	0
3	2	0	3	2	0	3	2	0	3	2	0
3	3	0	3	3	0	3	3	0	3	3	0
3	4	0	3	4	0	3	4	0	3	4	0
3	5	0	3	5	0	3	5	0	3	5	0
3	6	0	3	6	0	3	6	0	3	6	0
3	8	0	3	8	0	3	8	0	3	8	25
3	9	0	3	9	0	3	9	0	3	9	0
4	2	0	4	2	25	4	2	0	4	2	0
4	3	0	4	3	25	4	3	0	4	3	0
4	4	0	4	4	0	4	4	0	4	4	0
4	5	0	4	5	25	4	5	0	4	5	0
4	6	0	4	6	25	4	6	0	4	6	0
4	8	0	4	8	0	4	8	0	4	8	25
4	9	0	4	9	25	4	9	0	4	9	0
5	2	3,200	5	2	1.600	5	2	1,600	5	2	800
5	3	1,600	5	3	1,600	5	3	1.600	5	3	800
5	4	3,200	5	4	1,600	5	4	1,600	5	4	800
5	5	0	5	5	0	5	5	0	5	5	0
5	6	25	5	6	25	5	6	50	5	6	50
5	8	800	5	8	800	5	8	1,200	5	8	800
5	9	50	5	9	50	5	9	50	5	9	25
6	2	1,600	6	2	3,200	6	2	1,600	6	2	800
6	3	1,600	6	3	3,200	6	3	1,600	6	3	800
6	4	1,600	6	4	3,200	6	4	1,600	6	4	800
6	5	50	6	5	25	6	5	50	6	5	25
6	6	0	6	6	0	6	6	0	6	6	0
6	8	800	6	8	1,200	6	8	1,200	6	8	1,200
6	9	25	6	9	50	6	9	25	6	9	25
8	2	0	8	2	0	8	2	0	8	2	0
8	3	0	8	3	0	8	3	0	8	3	0
8	4	0	8	4	0	8	4	0	8	4	0
8	5	0	8	5	0	8	5	0	8	5	0
8	6	0	8	6	0	8	6	0	8	6	0
8	8	0	8	8	0	8	8	0	8	8	0
8	9	0	8	9	0	8	9	0	8	9	0
9	2	800	9	2	3,200	9	2	800	9	2	800
9	3	800	9	3	3,200	9	3	800	9	3	800
9	4	800	9	4	3,200	9	4	800	9	4	800
9	5	25	9	5	100	9	5	50	9	5	25
9	6	25	9	6	200	9	6	25	9	6	0
9	8	1,600	9	8	1.200	9	8	400	9	8	1,200
9	9	0	9	9	0	9	9	0	9	9	0
Unabsorbed	2	3,200	Unabsorbed	2	3 200	Unabsorbed	2	1,600	Unabsorbed	2	800
Unabsorbed	3	3,200	Control	3	3,200	Control	3	1,600	Unabsorbed	3	800
Control	4	3,200	Control	4	3,200	Control	4	1,600	Control	4	800
Control	5	50	Control	5	100	Control	5	50	Control	5	25
Control	6	25	Control	6	200	Control	6	50	Control	6	50
Control	8	1,600	Control	8	1,200	Control	8	1.200	Control	8	1,200
Control	9	50	Control	9	100	Control	9	50	Control	9	25

strains (5, 6 and 9) were selected. Absorption of serums 2, 3, 4 and 8 was carried out with these 7 strains as described, and the selected strains were then agglutinated with the absorbed serums. The results of these experiments are shown in table 2.

From a study of this table it is seen that when serum 2 was absorbed with the homologous strain, not only were the agglutinins for that strain removed, but likewise the agglutinins for all the other strains tested, and when it was absorbed with strains 3, 4 and 8, which agglutinated with it in high dilution, likewise all agglutinins for the other strains were removed. When, however, the serum was absorbed with strains 5, 6 and 9, which were not originally agglutinated by it in high

TABLE 3

DIRECT AGGLUTINATION OF STRAINS OF BACILLUS INFLUENCE WITH ANTI-B. INFLUENZAE SERUM OBTAINED FROM DR. BELL

Agglutination at 56 C. for 18 hours

Strains	Serum												
	B. Ous-lander	Burds-ley	Dock	Cot-ton	198	Bell	Fergu-son	Shinde-wolf	Par-sons	Ger-hart	Esser-mann	Bun-yard	Laser-sohn
1	0	0	25	0	0	0	0	0	0	0	0	0	0
2	25	50	100	200	100	0	0	0	400	25	0	0	200
3	0	50	100	200	100	0	0	0	400	25	0	0	200
4	0	50	100	50	100	0	0	0	200	50	0	0	100
5	0	0	200	200	0	0	0	0	0	0	0	0	0
6	0	0	100	0	0	0	0	0	0	0	25	0	0
8	0	0	50	400	0	0	0	0	25	0	0	0	0
9	0	0	50	0	200	0	0	0	25	0	50	0	0
10	0	25	200	50	0	100	50	0	0	100	0	50	25
11	0	0	200	0	0	0	200	0	0	0	0	0	0
12	0	0	400	0	0	0	0	0	0	0	0	0	0
Homo-logous strain	1,200	1,600	3,200	1,200	800	1,600	1,600	3,200	3,200	3,200	1,600	3,200	1,600

dilution, the specific agglutinins for the absorbing organism were removed, whereas those for the other organisms were either not removed or were reduced by an insignificant amount.

Analogous results were encountered when serum from strains 3, 4 and 8 was absorbed, except that in the case of serum 3 and serum 8 the absorbed serum still agglutinated other strains in a dilution of 1 : 25 as contrasted with dilutions of from 1 : 1200 to 1 : 3200 previous to absorption. Cross agglutination after absorption, therefore, bore out previous agglutination results and indicated that in the case of strains 2, 3, 4 and 8 we were dealing with strains that were identical from an immunologic standpoint.

It seems evident, therefore, from these experiments that among the 12 strains studied 4 were encountered which were capable of being

placed in one common immunologic group on the basis of agglutination and absorption tests, and that the remaining 8 gave no evidence which would justify placing them in immunologic groups.

Opportunity was afforded, through the kindness of Dr. Bell, to carry out a series of agglutinations with serum obtained by immunizing rabbits with strains of influenza bacilli. Thirteen such specimens were studied, and the results are shown in table 3. This table shows that 13 specimens of anti-serum derived from another source failed to agglutinate in high dilution any of the strains originally isolated by us, thus indicating that the strains used by Dr. Bell for obtaining these serums were not closely related to ours from an immunologic standpoint.

DISCUSSION

If B. influenzae of Pfeiffer is the etiologic agent of epidemic influenza, then, according to Park,[2] in view of the rapidity of the spread of this disease, we must assume that we are dealing with a strain of this organism which has a very high invasive power for man. That the "virus" of influenza may increase in invasive power within a short space of time is entirely conceivable. In a report [11] on an epidemic of influenza occurring in an isolated post in the American Expeditionary Forces we found evidence in support of this view. In this particular epidemic successive bodies of troops hitherto free from influenza were exposed to the disease and the incidence of the disease was greater in each succeeding body of troops, even when precautions had been taken to destroy any virus that might have been left behind by the preceding group of men.

If, however, a strain or several strains of B. influenzae acquire increased virulence to the extent that they may cause large proportions of the exposed population to come down with the disease in a short period of time, it is scarcely conceivable that such strains would not show close immunologic relationship. As Park has emphasized, we should expect to find in any group of patients during the acute stage of influenza a large number of strains of the influenza bacillus showing identity from an immunologic standpoint. It is true that the epidemic-producing strain might be missed, but the work of Valentine and Cooper,[7] and of Povitzky and Denny [9] showed that strains of this organism isolated from a single patient were almost uniformly identical in their antigenic behavior.

[11] Chesney, A. M., and Snow, F. W.: Jour. Lab. & Clin. Med., 1920, 6, p. 78.

This study of 12 strains isolated from patients during the recurrent epidemic of 1920 demonstrates that 4, or 33⅓% were identical, and the remaining 8 gave no evidence of close immunologic relationship. When the study was completed, it was found by reference to the records of the laboratory that 2 of them, Nos. 3 and 4, had been isolated from the same patient, one (3) by nasopharyngeal culture, the other (4) by inoculation of sputum into mice. This fact was not known at the time the experiments were being carried out. When it is taken into consideration, however, the percentage of identical strains encountered in 11 different patients drops to 27.

We are inclined to consider that this percentage is too small to be of any real significance from the standpoint of etiological relationship of the disease. Although it is not beyond the realms of conjecture that these identical strains which we isolated are representatives of a strain responsible for the epidemic, we are far from claiming that such is the case. We think that a far greater percentage of identical strains should be obtained before concluding that this organism bears a causal relationship to epidemic influenza. We are therefore in substantial agreement with the view of those who hold that in the case of influenza bacilli we are dealing with a group of heterogeneous organisms, some of which may be related immunologically, but that the majority of them, even when obtained from influenza patients, are not capable of being placed in one immunologic group.

It was the intention, before reporting this work, to study these strains from the standpoint of their biochemical reactions as has been done by Jordan,[12] and Stillman and Bourn,[13] but through an unfortunate accident 9 of the strains were lost, and this fact necessitated the abandonment of the work at this point.

SUMMARY AND CONCLUSIONS

Twelve strains of hemoglobinophilic bacilli obtained from 11 patients with influenza during the recurrent epidemic of 1920 have been studied immunologically.

Cross-agglutination tests and absorption experiments indicate that of the 12 strains 4, or 33⅓% were identical in their immunologic reactions.

No evidence of relationship to strains obtained from another source was encountered.

[12] Jour. Am. Med. Assn., 1919, 72, p. 1542.
[13] Jour. Exper. Med., 1920, 32, p. 665.

The influenza bacillus is a representative of a heterogeneous group of organisms possessing the common property of requiring hemoglobin for growth but differing in their antigenic properties, although immunologically identical strains may occur in the same patient and have been found in 27% of a small series of cases.

These results lend no support to the view that the influenza bacillus of Pfeiffer is the cause of epidemic influenza.